Green moulds and bacterial blotch of the cultivated mushroom, *Agaricus bisporus*

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Abstract: Green moulds and bacterial blotch affect the production and quality of commercial mushrooms (*Agaricus bisporus*) in Canada. The University of Guelph and Brock University have collaboratively sought to understand these diseases and assist the mushroom growers of Canada through applied research. *Trichoderma aggressivum* f. *aggressivum*, a green mould, directly reduces mushroom yield through growth inhibition. The extent of yield loss is dependent on host strain present; brown strains are less affected than white strains. Extracellular chitinases and volatile antifungal metabolites produced by *T. aggressivum* have been implicated as inhibitory factors. Exhaustive sanitation and hygiene management limit the spread of the disease. Chemically, green mould disease can only be managed preventatively by coating the spawn grains with benzimidazole fungicides. Other *Trichoderma* species affect mushroom quality by spotting the mushrooms prior to or after harvest. The benzimidazole fungicides are efficacious in lowering the endemic *Trichoderma* populations in the casing materials. Bacteria principally affect mushroom quality. In Canada several distinct *genotypes* with the ability to cause various blotch symptoms have been identified through molecular methodologies. Some bacterial genotypes at low concentrations cause unique post-harvest spotting symptoms. Other non-Pseudomonas species have also been identified as causal agents of blotch symptoms.

Key words: Green moulds, Trichoderma spot, bacterial blotch, *Agaricus bisporus, Trichoderma aggressivum* f. *aggressivum, Pseudomonas tolaasii,* chitinases, volatile antifungal metabolites, benzimidazole fungicides

1 Introduction

Viral, fungal and bacterial diseases affect commercial mushrooms (*Agaricus bisporus*). These diseases may either reduce mushroom yield or its quality. Symptoms of virus diseases (La France and mushroom virus X) may consist of simply a slight reduction in mushroom yield, bare unproductive areas, and off-coloured and malformed sporophores.[1, 2] The principal fungal diseases, *Verticillium fungicola, Mycogone perniciosa* and *Cladobotryum dendroides,* malform and engulf the mushroom or spot the pileus.[3] The fungus, *Trichoderma aggressivum* f. *aggressivum,* destroys the growing medium resulting in no mushroom production, and engulfs or spots the mushroom.[4] Other *Trichoderma* species simply infect the pileus lowering quality.[5, 6] Bacteria may distort and reduce yield or may spot the sporophores.[7]
The mushroom research program at the branch campus of the University of Guelph (Vineland Station, Ontario) together with Brock University (St. Catharines, Ontario) have collaboratively sought to understand some of these diseases and assist the mushroom growers of Ontario. This paper summarizes our recent efforts against Trichoderma green mould (*Trichoderma aggressivum* f. *aggressivum*), Trichoderma spot (*Trichoderma* spp.) and bacterial blotch (*Pseudomonas tolaasii*).

2 Trichoderma Green Mould

Trichoderma green mould [*Trichoderma aggressivum* f. *aggressivum*, formerly known as *Trichoderma harzianum* (biotype Th4)[8]] has been a destructive pathogen in the Canadian mushroom industry since 1990[4] and its European counterpart [*Trichoderma aggressivum* f. *european* = *Trichoderma harzianum* (biotype Th2)] since 1985.[9] Practical farm management of this disease has been accomplished through aggressive sanitation and hygiene practices,[10] coating of spawn grains with benzimidazole fungicides[11, 12] and judicious use of growing materials.[13, 14]
The presence of *T. aggressivum* renders the compost totally unproductive; whereas, other *Trichoderma* species permit mushrooms to grow within infested areas and along side of visible *Trichoderma* colonies. The mechanisms that account for these differences within genera are unknown. Volatile antifungal compounds and chitinase enzymes have been identified as contributors to this differential effect on *Agaricus bisporus*.

2.1 Antifungal compounds

Compounds extracted from liquid cultures of *T. aggressivum* and *T. harzianum* (biotype Th1) inhibited *A. bisporus* growth (Sylvan strain S130, Sylvan Spawn Company, Kittanning, PA). Crude extracts of *T. harzianum* produced zones of inhibition to *A. bisporus* on agar greater than 2.5 times that of the *T. aggressivum* f. *aggressivum*.[15]
Purification of the *T. aggressivum* crude extract, using thin layer chromotography, yielded two chromophores with Rf values of 0.56 and 0.79. Both of these purified compounds inhibited the growth of *A. bisporus* similar to the crude extract from liquid culture. Neither of these chromophores was detected from the typical *T. harzianum;* however, a different chromophore with R_f value of 0.72 was observed.[15]
Extraction of metabolites from *T. aggressivum* infected compost, using a protocol similar to the liquid culture extraction, yielded only the R_f 0.56 compound. Uninfected composts did not yield either compound.[15]
Rinker and Alm[16] reported mushroom compost spawned with brown strains produced significantly more mushrooms than compost spawned with white strains when the composts were infested with *T. aggressivum.* Extracts from liquid cultures of an *A. bisporus* brown strain (Sylvan strain SB65) produced significantly smaller zones of inhibition than a white strain (Sylvan strain S130).[15]
Silica column chromatography was used to purify the major chromophore (R_f 0.79) from the liquid culture. GC/MS analysis revealed that the compound has a molecular formula of $C_{18}H_{10}O_3$ and when compared to a database of standard compounds, the compound was designated as 3,4-dihydro-8-hydroxy-3-methylisocoumarin.[15]

2.2 Chitinase enzymes

The antagonistic mechanisms, by which *Trichoderma* species parasitize other fungi, include competition for space and nutrients, antibiosis and release of hydrolytic enzymes. Since *T. aggressivum* grows more rapidly than *A. bisporus,* compost fully colonized by *A. bisporus* is less easily colonized by *T. aggressivum* (less competitive) than freshly spawned compost.[13] Although antibiotics have not been demonstrated in *T. aggressivum, Trichoderma* species do produce volatile and non-volatile compounds which arrest the growth and development of fungal species.[17-19] Krupke *et al.*[15] demonstrated that volatile compounds from *T. aggressivum* and *T. harzianum* reduced the growth of *A. bisporus*. Haran *et al.*[20] noted that *T. harzianum* excretes lytic enzymes such as proteases, glucanases and chitinases that degrade the fungal cell wall. It is the contribution of the chitinase enzymes in the destructive nature of *T. aggressivum* to *A. bisporus* that has further occupied our research efforts.
Brown and white mushroom strains have demonstrated differences both *in vivo*[16] and *in vitro*[15] in their responses to *T. aggressivum.* Guthrie (2003)[21] characterized the chitinase enzyme activities within selected brown

strains [Amycel 2400 (Amycel Spawn Company, Watsonville, CA) and Sylvan SB65 (Sylvan Spawn Company, Kittanning, PA)], white strains [Horst U1 and Sylvan 130 (Sylvan Spawn)] and *Trichoderma aggressivum* f. *aggressivum* (University of Guelph Culture T586). Measurements of chitinolytic activity within solitary cultures showed that brown strains were substantially greater than white strains. For example, specific intracellular chitinase activities of the Amycel 2400, Sylvan SB65, Horst U1 and Sylvan 130 were 3.78, 1.82, 0.85 and 0.43 U μg^{-1}, respectively. [A unit (U) of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 ng of 4-methylumbelliferone min^{-1} with the specific activity defined as number of units of enzyme activity per microgram of crude protein[21, 22]] Similar differences, but less dramatic, were observed for the extracellular chitinase activities, 3.80, 1.92 and 1.50 (two enzymes), 1.06, and 1.19 and 1.21 U μg^{-1} (two enzymes), respectively. The chitinase type for both the intracellular and extracellular enzymes of the solitary cultures of the brown and white strains was *N*-acetylglucosaminidase. In solitary cultures of *T. aggressivum*, the intracellular chitinase activity of two enzymes were 0.65 and 0.85 U$\mu g^{-1}$and that of the extracellular enzyme 2.92 U μg^{-1}. The chitinase types for the solitary culture of *T. aggressivum* were *N*-acetylglucosaminidase and endochitinase for the intracellular isolations and a chitobiosidase for the extracellular isolations.

Measurements of chitinase activities of dual cultures of *T. aggressivum* and *A. bisporus* were recorded over a 14-day period. Analyses revealed four intracellular enzymes: *N*-acetylglucosaminidases (122 kDa and 96 kDa), a 36 kDa endochitinase and a 40 kDa chitobiosidase. On the other hand, extracellular protein analyses of dual cultures detected three chitinases: the 122 kDa and 96 kDa *N*-acetylglucosaminidases and a 40 kDa chitobiosidase. During the first 6 days, the activity of 122 kDa increased in activity. However, as the presence of the 96 kDa band increased in activity, the 122 kDa decreased. The brown strains had the highest levels of the 96 kDa *N*-acetylglucosaminidase; whereas, the white strains produced the 96 kDa enzyme at consistently lower levels. The presence of higher levels of the 96 kDa *N*-acetylglucosaminidase in the brown strains may in part account for the greater tolerance of the brown strains to a *T. aggressivum* infection.

2.3 Chemical management of *Trichoderma aggressivum*

Trichoderma aggressivum is a disease that must be managed preventatively. The spores are sticky and thus quite portable. At the height of the Ontario epidemic, disease propagules could be isolated in nearly every conceivable location on an infested farm.[23] Exhaustive and extensive sanitation and hygiene have assisted in eliminating the threat on many farms.[10] Chemical treatment in response to the pathogen's presence is not effective.[24] However, preventative chemical treatment is efficacious. Coating the spawn grains with bendimidazole fungicides (benomyl, carbendazim and thiabendazole) reduced infection.[11, 12] This resulted in registration of carbendazim in the UK and benomyl in Canada as a spawn coating.

Benomyl was withdrawn from the market and has not been permitted for use on mushroom spawn in Canada since December 31, 2003. An alternative bendimidazole fungicide (thiophanate methyl) received emergency registration in Canada in October 2003. Efficacy data submitted for that registration were based on that generated by the USA researchers. Subsequent to that data submission, our program has generated additional data. Two trials with the same fungicide rates produced rather different results. In each trial, compost was challenged with *T. aggressivum* spores by placing these spores on spawn grains treated with benomyl [0.25 g (a.i.) in 22 g gypsum kg^{-1} spawn] and thiophanate methyl [0.44g or 88 g or 1.31 (a.i.) in 55 g gypsum kg^{-1} spawn; 0.5 x; 1.0 x and 1.5 x, respectively]. In both trials no green mould colonies were observed in the benomyl treated spawn. With respect to thiophanate methyl, in the first trial the 0.5x, 1x and 1.5x treatments showed 33, 50 and 0% infection by *T. aggressivum,* respectively. However, in the second trial, the infections were 0, 16 and 0%, respectively. The principal difference in the two trials was the compost moistures (68.5 and 65.9, respectively), which may account for the differences in susceptibility of compost. Rinker[4] reported differences in the response of compost to an infestation of *T. aggressivum.*

Rinker and Alm[13, 14] reported that mushroom supplements may aggravate a green mould infection and that if

the supplement was 'protected' with a fungicide the infection was reduced. Researchers (Pete Romaine and Dan Royse, personal communication) have observed that, by mixing thiophanate methyl with an 'unprotected' supplement or the combination of spawn and supplement treatment, the impact of a green mould infection on yield is reduced. However, in recent studies we found no control of a green mould infestation when thiophanate methyl was mixed with supplement at rates up to 1.5g kg^{-1} of supplement (1.5 times the efficacious rate reported by Romaine and Royse).

3 *Trichoderma* Species

The casing material is a harbinger of bacteria and fungi. Among the fungi are various *Trichoderma* species. Rinker and Alm[25] have identified at least 10 species of *Trichoderma* from commercial mushroom farm operations. These species do not create the havoc in production losses caused by *T. aggressivum.* However, they do affect mushroom quality during production or after harvest.[5, 6]

Application of chemicals to the casing material in the irrigation water is one method used to manage these casing *Trichoderma.* Benomyl, an effective chemical against the casing *Trichoderma*, was withdrawn from the market by the registrant in 2003. Our research program evaluated both *in vitro* and *in vivo* mushroom fungicides currently allowed, registered or proposed in Canada and the United States. These fungicide products included: benomyl, chlorothalonil, thiabendazole and thiophanate methyl. *In vitro* evaluations, where the fungicide was incorporated into cooling agar media, demonstrated that the different species of *Trichoderma* varied in their sensitivity to these fungicides and that the fungicide sensitivity, in general, was similar with benomyl, thiabendazole, and chlorothalonil, followed by thiophanate methyl. *In vivo* trials, where sphagnum casing was irrigated with the fungicides, showed that, generally, benomyl, thiabendazole and thiophanate methyl were effective in lowering the number of *Trichoderma* propogules in the casing; whereas, chlorothalonil was not.

4 Bacterial Blotch

Bacterial blotch reduces mushroom quality through pileus and stipe discoloration and pitting of the pileus. Cap spotting is principally attributed to *Pseudomonas tolaasii*. Wells *et al.*[26] in the USA, Godfrey *et al.*[27] in New Zealand and Dobbins[28] in Canada have reported novel symptoms caused by *P. tolaasii* strains. The identification of novel symptoms caused by a distinctively different pathovar of *P. tolaasii*[28] served as a basis to survey the Canadian mushroom industry for differences in bacteria causing blotch disease symptoms.

Blemished mushrooms, collected across Canada, showed that the causal agents for these symptoms were variants of *P. fluorescens, P. tolaasii* and two other bacterial species. All the isolates (170) collected were tested for pathogenicity by the mushroom rapid pitting test. Over two-thirds of the isolations produced yellow, brown and dark brown symptoms. The majority of isolates causing symptoms were identified through biochemical analysis, adapted from Sorenson *et al.*,[29] as *P. fluorescens.* All five biotypes caused blotch symptoms with 45% of the isolates consistent with biotype III. Three blotch causing organisms, not identified by the biochemical tests, were tentatively identified by fatty acid analysis as *P. putida, Cedecea davisiae* and *Serratia liquefaciens.* A dendrogram developed through amplified fragment length polymorphism (AFLP) analysis and neighbor joining analysis identified at least 5 major groups of *Pseudomonas.*[30, 31]

These results suggest that there is a substantial diversity of organisms responsible for blotch disease of the cultivated mushroom. However, investigations need to determine if the management strategies will differ substantially among these diverse blotch-causing organisms.

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